Fenretinide induces lethal autophagy via a novel ensemble of life and death regulators: dihydroceramide and sphinganine versus sphinganine 1-phosphate

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Undergraduate Thesis

Abstract

Sphingolipids are unique lipids that serve numerous functions important in cell regulation. Some categories of sphingolipids have also been implicated in the mechanism of action of cancer chemotherapeutic drugs, with the hypothesis being that these drugs are effective because they alter sphingolipid metabolic pathways to increase the production of ceramide, a sphingolipid known to induce cell death. Fenretinide (4-hydroxyphenylretinamide, 4HPR) is one such drug and has been thought to induce apoptosis through an increase of ceramide biosynthesis. However, recent research by W. Zheng *et.al 2006*., has shown that fenretinide instead increases dihydroceramide, which has traditionally been regarded as innocuous in cell signaling. This study has explored how fenretinide induces cell death via elevation of dihydroceramide and has discovered that dihydroceramide and fenretinide are both potent inducers of autophagy, a pathway for turnover of cell constituents that can also trigger type II programmed cell death. In addition, this analysis has found that after dihydroceramide induces the formation of autophagosomes, it seems to be further metabolized to sphinganine and sphinganine 1-phosphate as indicated by an increase in the quantity of these species and whether or not this results in cell death depends on the balance between these highly bioactive toxic (sphinganine) versus anti-apoptotic (sphinganine-1-phosphate) compounds. Thus, these studies suggest that the mechanism of fenretinide toxicity has many components:

elevation of dihydroceramide to induce autophagy as well as changes in sphinganine and sphinganine-1-phosphate, with the balance between the latter dictating if the autophagy is lethal.

Introduction

In order to understand the mechanism of fenretinide toxicity presented, the following key concepts will first be discussed: i) sphingolipids, ii) role of sphingolipids in regulating cell proliferation and cell death, iii) autophagy, iv) current opinions on autophagic cell death, and v) role of sphingolipids in autophagy..

Sphingolipids - Sphingolipids are a family of phospho- and glycolipids found in all eukaryotic and some prokaryotic organisms. They are comprised of a sphingoid base backbone that may be unmodified, N-acylated, N-methylated, phosphorylated, or incorporated into more complex sphingolipids, such as ceramide phosphoinositols (yeast), ceramide phosphoethanolamines (insects), and ceramide phosphocholines (mammals and other organisms) as well as simple to complex glycosphingolipids (Figure 1). The biophysical and biochemical properties of this backbone differentiate sphingolipids from other lipid species. For example, at a neutral pH the sphingoid backbone carries a net positive charge, which is rare among membrane lipids (1). This property may be used to explain the relative ease with which sphingoid bases move among membrane bilayers, allowing them to influence many aspects of both intracellular and extracellular signaling (2).

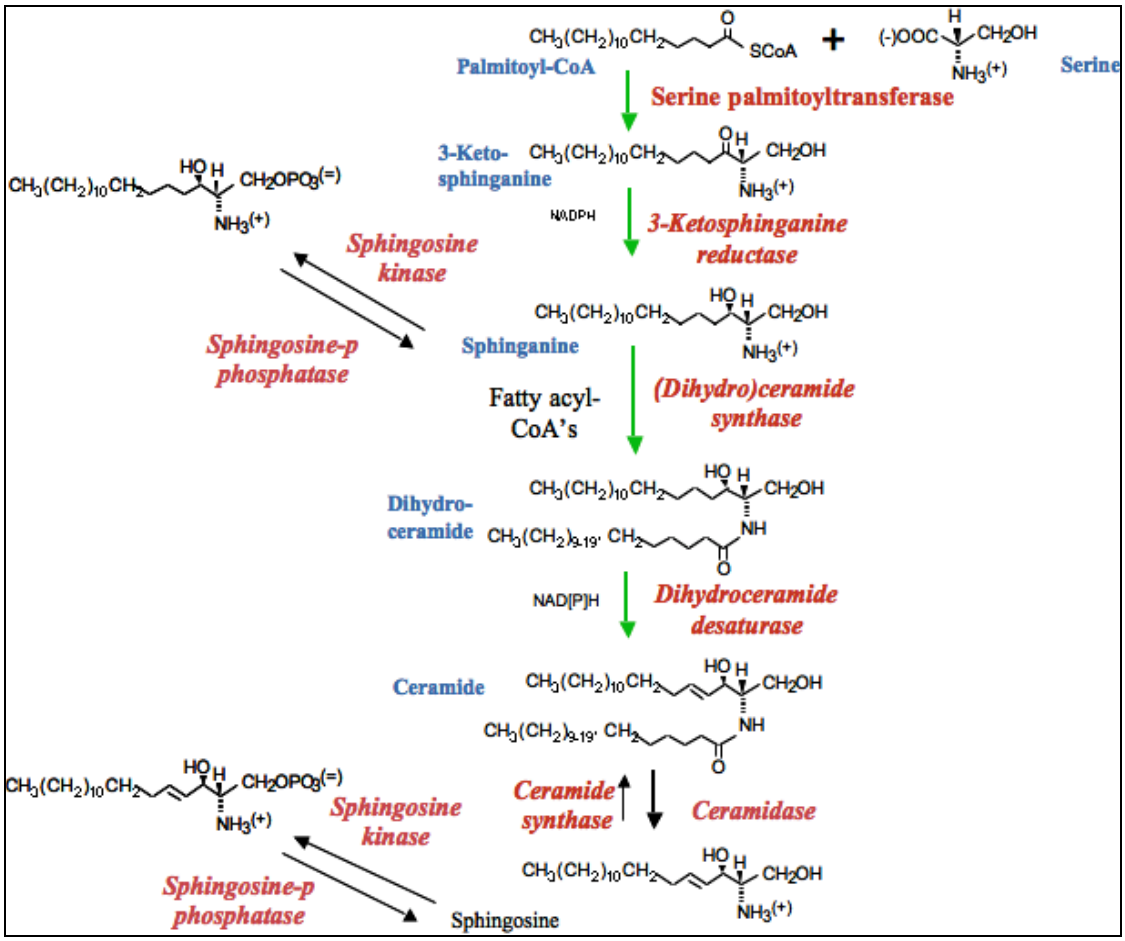


Figure 1. The sphingolipid metabolic pathway. Modified from source (3).

Figure 1 illustrates the complexity of the sphingolipid metabolic pathway depicting the enzymes involved and the intermediates generated as cells metabolize sphingolipids and modify the sphingoid base backbone. *De novo* sphingolipid biosynthesis begins with the condensation of serine and palmitoyl-CoA by serine palmitoyltransferase to yield 3-ketosphinganine. 3-ketosphinganine reductase then reduces 3-ketosphinganine to sphinganine, which is subsequently N-acylated by (dihydro)ceramide synthase yielding dihydroceramide (4). At this point, dihydroceramide desaturase introduces a 4,5 *trans* double bond into dihydroceramide forming ceramide (5). These steps take place on the cytosolic face of the ER (5,6). The

resulting ceramide can then be transported to the *cis* Golgi for incorporation into more complex sphingolipids (7).

Figure 1 also displays that this biosynthetic pathway has enzymes that function in both the synthesis and turnover pathways. Ceramidases, found in the organelles such as the lysosome and the mitochondria, could perform the reverse reaction of ceramide synthase and deacylate ceramide to form sphingosine (7). One such enzyme that is found in the lysosome is acid ceramidase; it not only converts ceramide to sphingosine in the biosynthetic pathway, but can also form sphinganine through the turnover of dihydroceramide (1,3). These backbone species, sphingosine and sphinganine may then be phosphorylated by sphingosine kinase to yield sphingosine- or sphinganine-1 phosphate, respectively. This complex metabolic pathway profoundly affects the cell, as changes in the natural rheostat of sphingolipid subspecies may serve as a determining factor in cell fate decisions.

Bioactive Sphingolipids - A delicate balance exists between the bioactive sphingolipids that activate cell survival and cell death pathways. Because new roles for sphingolipids are continuously being discovered, there is still much to be learned about how sphingolipids regulate many processes that occur within the cell. Traditionally, ceramide, sphinganine, and sphingosine have been considered to be the bioactive sphingolipids that induce cell death and growth arrest, while sphingosine- and sphinganine-1-phosphate have been regarded as inducers of cell survival and cell proliferation (2,8).

Sphingoid bases, sphingosine and sphinganine, are known to activate many signaling pathways that are cytotoxic to cells (2,9-11). Their ability to behave as

detergents when trapped in acidic vesicles allows them to induce cell death by acting as lysosomotrophic agents, which permeabilize the lysosomal membrane allowing lysosomal contents, such as proteases, to be released into the cytosol. These proteases inturn serve as upstream activators of well-characterized cell death pathways (12). Although sphinganine and sphingosine are cytotoxic to cells, when phosphorlyated by sphingosine kinase, they form sphinganine- or sphingosine-1 phosphate respectively, and these phosphorylated species promote cell proliferation rather than cell death (2,13).

Ceramide, one of the most widely studied sphingolipids, is a known inducer of apoptosis, type I programmed cell death. (8). Furthermore, the mechanism of action of certain chemotherapeutic drugs is hypothesized to result from alterations in the normal sphingolipid metabolic pathway to increase the biosynthesis of ceramide within the cell, which in turn results in apoptosis. Fenretinide, a chemotherapeutic agent currently in clinical trials for breast and many other types of cancers, is one such drug (14). Fenretinide is thought to increase ceramide by the coordinate activation of serine palmitoyltransferase and ceramide synthase (15,16). A second proposed mechanism for fenretinide's mode of action is thought to involve the production of reactive oxygen species (ROS), again resulting in the stimulation of the apoptotic pathway (17).

However, in previous studies to determine the effect of fenretinide on the *de novo* sphingolipid biosynthetic pathway, it was found that MCF7 breast cancer cells treated with fenretinide experienced only a nominal change in ceramide production; whereas, dihydroceramide production increased as much as 10-fold, both as the free species and as the backbone for dihydroceramide metabolites (2). The distinction between ceramides and dihydroceramides was possibly overlooked in previously published studies because

ceramide analysis was performed by techniques such as thin layer chromatography (TLC) or diacylglycerol kinase assays (DGK), and these techniques cannot readily distinguish dihydroceramides from ceramides. The mechanism of dihydroceramide accumulation has been investigated and appears to be due to the inhibition of dihydroceramide desaturase (2). This finding is significant because dihydroceramide has long been considered to be inactive in cell signaling (18). However, the ability of chemopreventive agents, such as fenretinide, to induce increases in dihydroceramide leads one to question the bioactive status of this molecule. Furthermore, this research identifies a previously uncharacterized signaling function for dihydroceramide, the induction of the formation of cytoplasmic vacuoles called autophagosomes.

Autophagy - Translated from the Greek meaning "to eat oneself," autophagy is an intracellular degradation and recycling pathway that is in various forms ubiquitous throughout eukaryotic cells. Macroautophagy, which will be referred to as autophagy for the remainder of this thesis, allows cells to degrade cytoplasmic organelles and proteins to produce amino acids, lipids, sugars, and nucleotides during nutrient poor conditions (19,20). Autophagy also provides a way for cells to degrade damaged organelles, thus helping cells survive in otherwise cytotoxic situations (21). In recent years mechanisms for monitoring the dynamic process of autophagy have provided evidence that this cell survival pathway can also result in programmed cell death, which will be discussed in more depth in the following section (22-24).

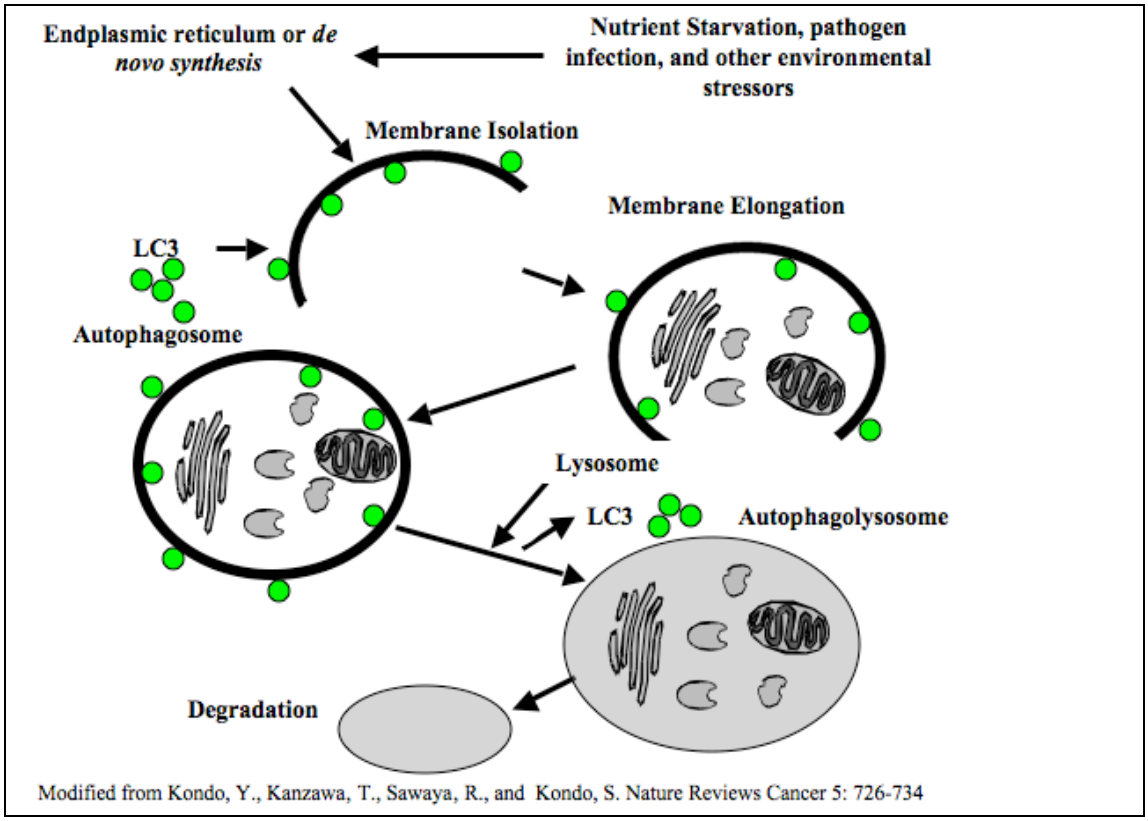


Figure 2. Process of Autophagy. Modified from source (25)

As seen in Figure 2, autophagy is usually triggered by one of the following events: nutrient starvation (26), bacterial or viral invasion (27,28), or certain anticancer drugs (29-31). Autophagy is mediated by a wide number of signaling pathways, and as technologies for monitoring and understading autophagy improve, many more are being elucidated (20). Autophagy begins with the isolation of a piece of the endoplasmic reticulum or a membrane created from de novo synthesis. This isolated double membrane elongates and recruits microtubules-associated protein light chain 3 (LC3) to its surface as it surrounds proteins and organelles to be degraded forming the autophagosome. The autophagosome subsequently fuses with the lysosome to form the autophagolysosome,

whose acidic environment allows for the degradation of the sequestered proteins and organelles (25).

Autophagy and Cell Death - Autophagy is a complex process; in some cases autophagy serves a protective role, but in other situations autophagy can result in cell death. Autophagic cell death, referred to as type II programmed cell death, is distinct from apoptosis, type I cell death, and necrosis, type III cell death. Degradation of organelles is one of the first events to occur during autophagy, while the cytoskeleton is one of the last items degraded prior to cell death. Whereas, during apoptosis, the cytoskeleton collapses almost immediately following induction and the organelles are among the last items degraded prior to cell death. Furthermore, necrotic death is characterized by an inflammatory response, not present in either autophagy or apoptosis (22). However, the distinction between autophagic cell death and apoptotic cell death is not always so distinct. There are many shared molecular signaling pathways activated during both autophagic and apoptotic cell death (20,32).

Currently a debate exists over autophagy's connection to cell death, with questions surrounding the idea of whether autophagy alone induces cell death or whether cell death processes, like apoptosis, play a role in autophagic cell death. Some believe that autophagy triggers apoptosis, while others believe that autophagy and apoptosis can occur simultaneously inside a cell (19-22,25). In a recent review, Yoshimori acknowledged that there is not an understanding of what autophagy-inducing conditions trigger cell death and what conditions trigger cell survival (24). No current hypotheses that explain protective and lethal autophagy can be generalized for all autophagy-inducing treatments (nutrient starvation, viral and bacterial infection, and

chemotherapeutic drug). It is possible that each autophagy-inducing treatment could contain its own molecular switch that signals when autophagy is protective or lethal (32,33).

Autophagy is directly linked to cancer and appears to regulate both cell survival and cell death (25). Several chemotherupeutic drugs have previously been identified as inducers of autophagy and/or autophagic cell death, including temozolomide in several malignant glioma cell lines (30) and tamoxifen in MCF7 breast cancer cells (29), Furthermore, this study also identifies the chemotherapeutic agent fenretinide as an inducer of autophagy and/or autophagic cell death. So, what mechanism do these and other chemotherapeutic agents use to convert autophagy from a survival pathway to a death pathway? The ultimate goal of this research is to explain whether this life and death transition is dependent on the type of sphingolipid mediators produced inside the cell.

Sphingolipids Induce Autophagy - Because of their unique role in membrane organization and structure and their dual role as both cell survival and cell death mediators, sphingolipids appear to be unique mediators of autophagy. Furthermore, they could potentially be a bioactive mediator that controls the switch from protective to lethal autophagy.

Many studies have elegantly established ceramide's ability to mediate autophagy through the activation of several key molecules. Ceramide induces lethal autophagy through the activation of BNIP3, a mitochondrial death associated protein that induces autophagy (34). Ceramide also activates lethal autophagy by upregulating beclin1, a gene that is necessary for autophagy to occur in cells (29,34). Another sphingolipid mediator of autophagy, sphingosine-1-phosphate induces protective autophagy in MCF7 breast

cancer cells during times of nutrient starvation (35). We are currently investigating how other sphingolipid species, such as dihydroceramide, regulate autophagy following treatment with the chemotherapeutic agent, fenretinide. This thesis aims to provide more insight into the molecular basis of fenretinide's role in cell death via a novel ensemble of sphingolipid autophagic regulators, as well as, characterize a previously unidentified function of dihydroceramide.

Experimental Procedures

Reagents – C2- Ceramide and C2- Dihydroceramide were purchased from Avanti Polar Lipids Inc (Alabaster, AL). C2-Ceramide and C2-Dihydroceramide analogs contain a short acyl chain allowing for easy delivery into the cell when complexed with bovine serum albumin 1:1 or 2:1 respectively. Tamoxifen and fenretinide (4HPR, retinoic acid p-hydroxyanilide) were purchased from Sigma (St. Louis, MO). Myriocin (ISP1) was purchased from BioMol (Plymouth Meeting, PA). Acid ceramidase siRNA was purchased from Dharmacon (Lafayette, CO). The transfection reagent, DharmaFECT®1, was purchased from Dharmacon (Lafayette, CO). Dimethylsphingosine was purchased from Avanti Polar Lipids (Alabaster, AL). Martine Leipelt constructed the pEGFPC2-LC3A plasmid. The LC3A cDNA construct was purchased from OriGene (Rockville, MD). The pEGFPC2 vector was purchased from Clontech (Mountain View, CA).

Cell Lines - MCF7 cells were obtained from the ATCC (Manassas, VA) and cultured at 37° C in 5% CO_2 in Minimal Essential Media (MEM) supplemented with 10% FBS, 100x nonessential amino acids (0.01%), sodium bicarbonate (1.5 mg/mL), and bovine insulin (0.01 mg/mL).

Methods to Measure Autophagy - LC3 (microtubule-associated protein light chain 3) is normally diffusely distributed throughout the cytoplasm, but upon induction of autophagy is recruited to the autophagosomal membrane and appears in a punctate pattern. When LC3 is tagged with a fluorescent protein, its localization, in the cytoplasm or as a part of the autophagosome, can be easily monitored using confocal microscopy. For our studies, GFP-LC3 localization was monitored as previously described (23,26).

MCF7 cells were cultured on coverslips in a 24 well plate at a seeding density of 3.75×10^5 cells/well 24 hours prior to transfection (GeneJuice®, Novagen) with the GFP-LC3 plasmid. Following a 24-hour transfection, to allow for expression of the autophagic marker, cells were treated with the appropriate treatment as dictated by the experiment. Following treatment, cells were fixed using 2% formaldehyde for 12 minutes. The cell nuclei were stained with Hoecsht 33342 (Invitrogen, Carlsbad, CA) for 5 minutes at room temperature to clearly identify the number of cells for autophagy scoring purposes. Coverslips were examined using the Zeiss 510 Confocal Microscope (Thornwood, NY).

Confocal images were scored to determine the number of cells that contain GFP-LC3 punctuate vesicles. The following were used as criteria during the scoring process: 1) Cell patches of 70 or more cells were not scored due to the differing environments of periphery and interior cells, and 2) only cells with entire nuclei and cytoplasm in the image were scored.

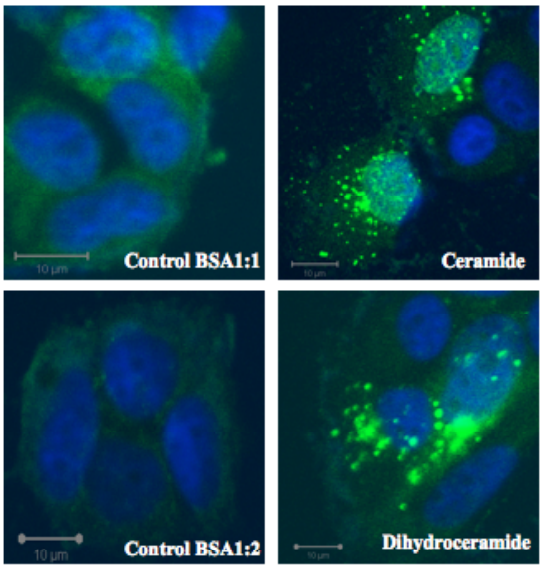
Cell Death Assay - Cell death was measured by the Wst-1 cell proliferation assay (Roche Molecular Biochemicals). MCF7 cells were cultured in a 96 well plate at a seeding density of 1×10^4 cells/well for 24 hours prior to treatment. Following treatment, the cells were treated with 10 μL of the Wst-1 reagent for one hour. Colormetric analysis

of the plate was then conducted at 450 nm using a microplate reader (Spectra Max Plus, Molecular Devices).

Results

Dihydroceramide and fenretinide induce autophagy - Autophagy was monitored in MCF7 cells transfected with the GFP-LC3 plasmid. Previous studies illustrated that exogenously added C2- ceramide is an inducer of autophagy (29,34), therefore in our studies C2- ceramide served as a positive control. Punctuate GFP-LC3 autophagosomes were observed when cells were treated with C2- ceramide (25 μM, 24 hours). Quantitatively, 17% of the total cells scored contained autophagosomes (Figure 3). Surprisingly, when MCF7 cells were treated with C2- dihydroceramide (25 μM, 24 hours), autophagy was also observed (Figure 3), and 16% of the scored cells contained GFP-LC3 tagged autophagosomes. As can be seen in Figure 3, MCF7 cells treated with C2- dihydroceramide resulted in an approximate 4-fold increase in autophagy when compared to its respective BSA control (2:1).

The formation of punctate autophagosomal vesicles following treatment with exogenous C2- dihydroceramide identifies a previously uncharacterized signaling function for this previously reported inert molecule. Furthermore, the ability of C2-dihydroceramide to induce autophagosome formation prompted us to investigate the ability of fenretinide (4HPR) treatment to induce autophagosome formation in MCF7 cells, since, as previously discussed, 4HPR treatment leads to an increase in dihydroceramide in MCF7 cells (2).

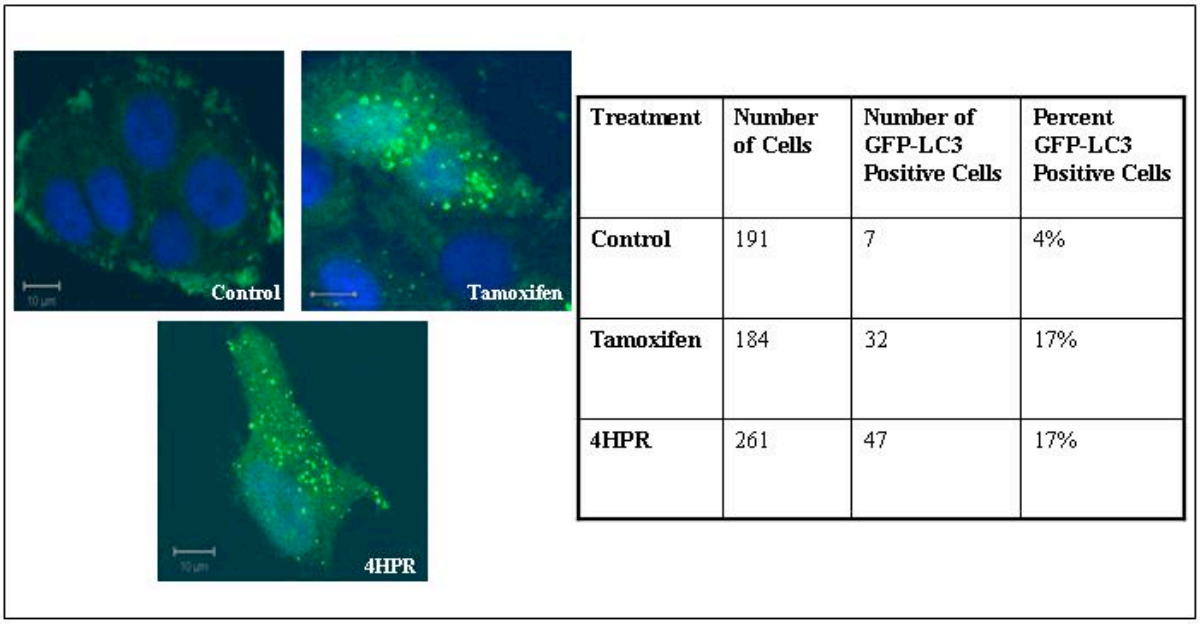


Treatment	Number of Cells	Number of GFP-LC3 Positive Cells	Percent GFP-LC3 Positive Cells
BSA Control 1:1	318	12	4%
BSA Control 1:2	278	14	5%
Ceramide	261	47	17%
Dihydro-ceramide	429	70	16%

Figure 3. Effect of dihydroceramide on autophagy. Cells were cultured for autophagy analysis as described in "Experiment Procedures." Following transfection with the GFP-LC3 plasmid, MCF7 cells were treated with C2- Ceramide (25 μM) or C2- Dihydroceramide (25 μM), complexed with BSA in 1:1 or 2:1 ratio respectively for 24 hours. The punctuate vesicles mark the presence of autophagosomes. The images shown are representative samples of multiple independent experiments. Autophagic cells were quantified as described in "Experimental Procedures."

Autophagy was monitored in MCF7 cells treated with fenretinide as described above. Tamoxifen, another anti-cancer agent and known inducer of autophagy in MCF7 cells (36), served as the positive control in this experiment. As can be seen in Figure 4, cellular treatment with tamoxifen (1 μM) induced autophagy in 17% of observed cells after 24 hours. Furthermore, cellular treatment with 4HPR (10 μM) also resulted in the formation of autophagosomes (Figure 4). Following 24 hours of 4HPR (10 μM) treatment, 17% of scored cells contained autophagosomes, resulting in an approximate 4-fold increase in autophagy when compared to control cells. Therefore, our results not only confirm tamoxifen's ability to induce autophagy, but also identify 4HPR as a novel inducer of autophagy.

Taken together, these results suggest that 4HPR-mediated autophagy may be dependent on intracellular increases in dihydroceramide (2). Therefore, we next investigated whether sphingolipid biosynthesis was necessary for 4HPR-induced autophagy.



Treatment	Number of Cells	Number of GFP-LC3 Positive Cells	Percent GFP-LC3 Positive Cells
Control	191	7	4%
Tamoxifen	184	32	17%
4HPR	261	47	17%

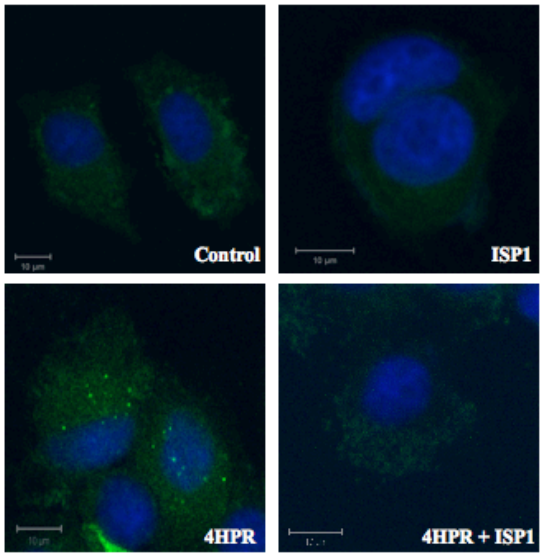
Figure 4. Effect of fenretinide on autophagy. Cells were cultured for autophagy analysis as described in "Experimental Procedures." Twenty-four hours after transfection with GFP-LC3 plasmid, MCF7 cells were treated with 4HPR (10 μM) or Tamoxifen (1 μM). The punctuate vesicles indicate the presence of autophagosomes. The images shown are representative samples of multiple independent experiements. Autophagic cells were quantified as described in "Experimental Procedures."

Sphingolipid biosynthesis is necessary for fenretinide-induced autophagy and fenreitnide cytotoxicity - ISP1 inhibits the first enzyme in the sphingolipid pathway, serine palmitoyltransferase, thus blocking *de novo* synthesis and subsequent dihydroceramide production (37). Treating cells with ISP1 allowed us to determine the effect of sphingolipid biosynthesis on 4HPR-induced autophagy. MCF7 cells treated with ISP1 (1 μM, 25 hours) alone did not result in the formation of autophagosomes (Figure 5a). In agreement with the previous finding, punctuate autophagosomes were observed following 4HPR treatment alone, with 13% of scored cells containing autophagosomes.

In contrast, MCF7 cells pre-treated with ISP1 (1 μM, 25 hours) for one hour prior to 4HPR (10 μM, 24 hours) treatment resulted in reduced levels of autophagy, in which only 5% of the cells contained autophagosomes, and this finding was consistent with the basal level of autophagy found in control cells (Figure 5a). These results led us to conclude that *de novo* sphingolipid biosynthesis, and possibly the production of dihydroceramide is necessary for 4HPR-induced autophagy.

The requirement of sphingolipids biosynthesis in 4HPR-induced autophagy (Figure 5a) prompted us to investigate whether the cytotoxicity of 4HPR was also dependent on sphingolipid biosynthesis. Cell viability analysis revealed that 4HPR treatment alone resulted in a 22% decrease in cell viability after 48 hours. However, upon the inhibition of sphingolipid biosynthesis with ISP1 pre-treatment, cell viability decreased only 7% following 48 hours of 4HPR treatment (Figure 5b). Taken together, these results suggests that *de novo* sphingolipid biosynthesis is necessary for both 4HPR-induced cell death and autophagy.

A.



Treatment	Number of Cells	Number of GFP-LC3 Positive Cells	Percent GFP-LC3 Positive Cells
Control	191	7	4%
ISP1	302	12	4%
4HPR	298	35	12%
4HPR+ ISP1	342	17	5%

B.

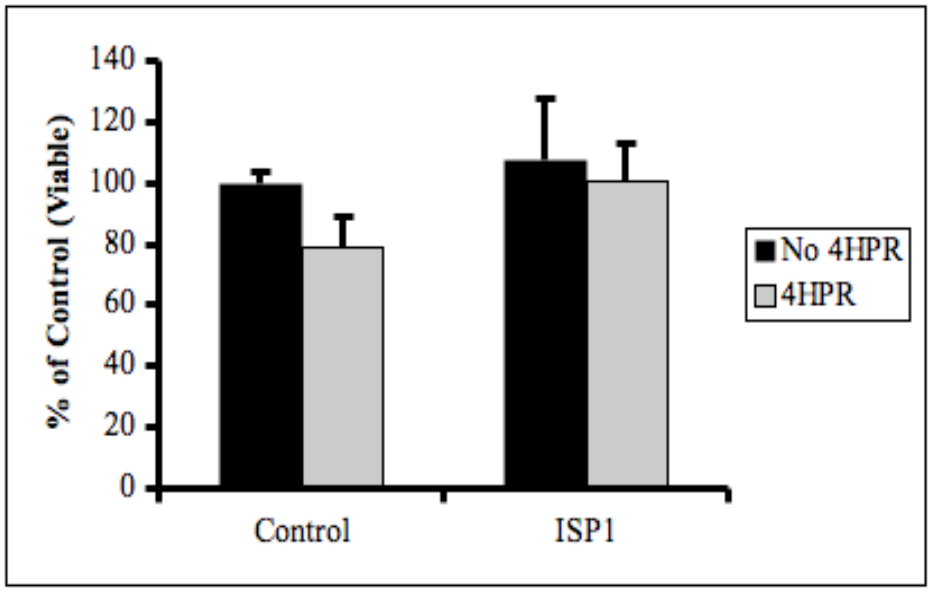


Figure 5. Sphingolipid de novo synthesis is necessary for fenretinide induce autophagy and cause cell death. A. Cells were cultured for autophagy analysis as described in "Experimental Procedures." Twenty-four hours after transfection, cells were pre-treated with ISP1 (1 μM) for 1 hour prior to treatment with 4HPR (10 μM). The punctuate vesicles indicate the presence of autophagosomes. The images shown are representative of multiple independent experiments Images were scored as described in the "Experimental Procedures."

B. Cells were for viability analysis as described in 'Experimental Procedures.' MCF7 cells were pre-treated with ISP1 (1 μM) for 1 hour prior to treatment with 4HPR (10 μM). Forty-eight hours after 4HPR treatment, Wst1 reagent was added and colormetric analysis was performed following one-hour incubation at 37ºC. All measurements have been normalized to control cells.

Dihydroceramide turnover in the autophagolysosome contributes to fenretinide cytotoxicity.- After fenretinide induced dihydroceramide accumulation mediates the formation of autophagosomes, dihydroceramide seems to be further metabolized to sphinganine and sphinganine 1-phosphate, as indicated by an increase in the quantity of these species in a previous study (2). The cytotoxicity of 4HPR is hypothesized to depend on the balance between these highly bioactive toxic (sphinganine) versus anti-apoptotic (sphinganine-1-phosphate) compounds.

Sphinganine may be produced from the hydrolysis of the accumulated dihydroceramide by acid ceramidase within the autophagolysosome, which presumably acquired lysosomal sphingolipids hydrolases upon autophagosomal and lysosomal fusion (37). Since sphinganine is known to be a toxic, lysosmotrophic agent (11,12), accumulation of this sphingoid base should result in 4HPR cytotoxicity or lethal autophagy. Furthermore, if sphinganine accumulation is due to the turnover of dihydroceramide rather than *de novo* synthesis and if sphinganine is involved in 4HPR cytotoxicity, then suppression of acid ceramidase should decrease the cytotoxicity of 4HPR.

In order to investigate this idea, MCF7 cells were transfected with acid ceramidase siRNA to suppress acid ceramidase activity prior to 4HPR treatment, and cell viability was assessed. Figure 6 displays cellular transfection with acid ceramidase siRNA for 72 hours alone results in slight background cell death, when compared to control cells that were not transfected. Control cells subjected to 4HPR (10 μM, 48 hours) treatment experienced appreciable cell death, with a 55% decrease in cell viability (Figure 6). In contrast, cells transfected with acid ceramidase siRNA (72 hours) prior to

4HPR (10 μM, 48 hours) treatment only experienced a 16% decrease in cell viability (Figure 6). These results suggest that the sphinganine accumulation observed in a previous study is indeed due to the turnover of dihydroceramide, which inturn contributes to the cytotoxic mechanism of 4HPR.

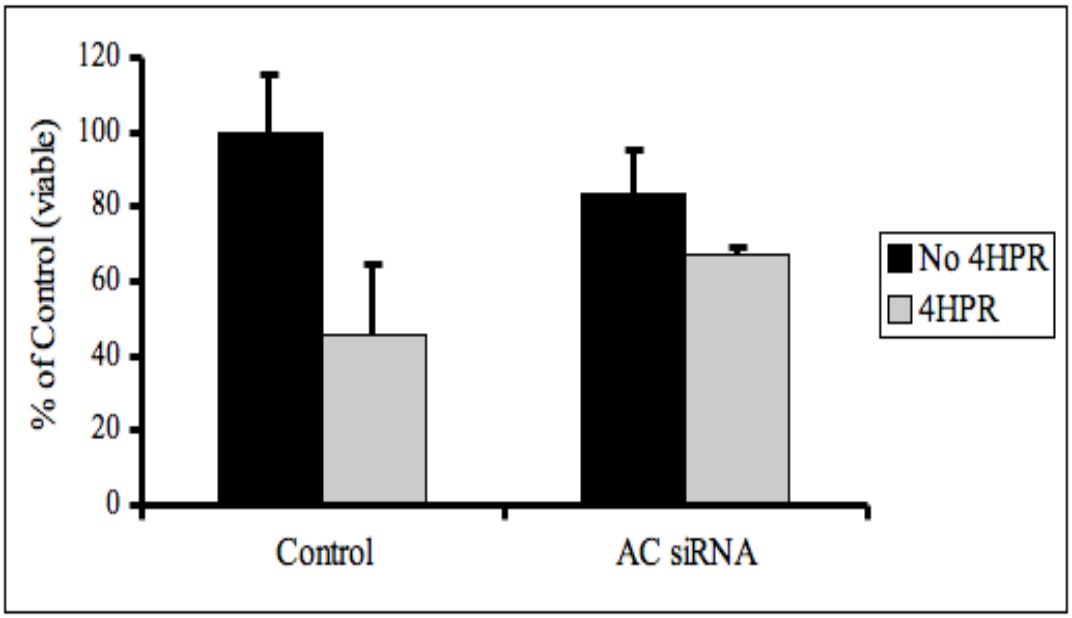


Figure 6. Acid ceramidase accounts for a portion of fenretinide cytotoxicity.
Cells were cultured for cell viability as described in "Experimental Procedures." MCF7 cells were transfected with acid ceramidase (AC) siRNA 24 hours later. 24 hours after transfection cells were treated with 10μM fenretinide. 48 hours after fenreitnide treatment and 72 hours after AC siRNA Wst1 reagent was added and colormetric analysis was performed following one hour incubation at 37ºC. All measurements have been normalized to control cells.

Inhibition of sphingosine kinase increases fenretinide cytotoxicity – A portion of the sphinganine produced from dihydroceramide turnover, described above, will presumably efflux from the autophagolysosome in a manner analogous to the lysosomal degradation of sphingolipids (2). Once effluxed, a portion of this sphinganine will become phosphorylated by sphingosine kinase to yield sphinganine 1-phosphate (13), owing to the accumulation of this species as noted in a previous study (2). A previously published study by Lavieu *et. al*. found sphingosine 1-phosphate to be an inducer of protective autophagy during conditions of nutrient starvation (35). Therefore, if

phosphorylation and removal of sphinganine to produce of sphinganine 1-phosphate is protective, then inhibition of sphingosine kinase and protective autophagy should increase the cytotoxicity of 4HPR.

In order to investigate this idea, dimethylsphingosine (DMS) was used to inhibit sphingosine kinase (38). MCF7 cells treated with 4HPR (10 μM, 48 hours) or DMS (1 μM and 2.5 μM, 48 hours) alone experienced an approximate 35% decrease in cell viability when compared to control cells (Figure 7). However, when MCF7 cells were pre-treated with DMS for 1 hour prior to 4HPR treatment, a more rapid, pronounced decrease in cell viability was observed, resulting in a 59% and a 77% decrease in cell viability following 1 μM and 2.5 μM DMS pre-treatments respectively (Figure 7). These results suggest that as sphingosine kinase is inhibited, protective autophagy may also be inhibited and result in a 4HPR induced lethal autophagic death.

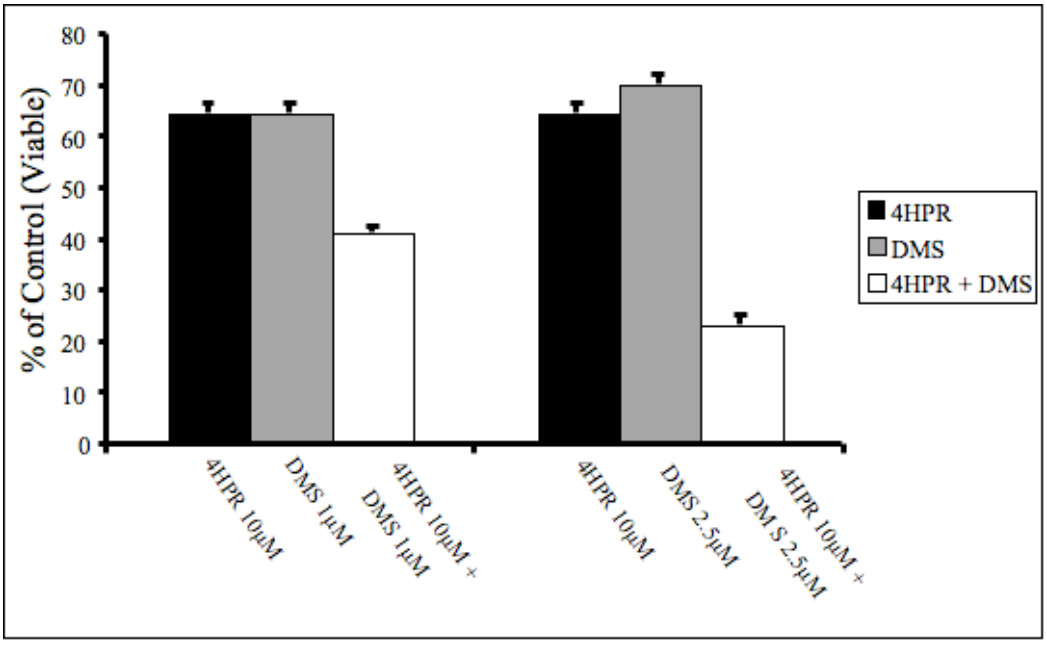


Figure 7. Inhibition of sphingosine kinase increases the cytotoxicity of fenretinide.
Cells were cultured for cell viability as described in 'Experimental Procedures.' Cells were treated with 4HPR (10 μM) alone, dimethlysphingsone (DMS) alone (1 μM or 2.5 μM) or pre-treated with DMS (1 μM or 2.5 μM) for one hour prior to treatment with 4HPR (10μM). 48 hours after 4HPR treatment Wst1 reagent was added and colormetric analysis was performed following one hour incubation at 37ºC. All measurements have been normalized to control cells.

Discussion

Fenretinide is a promising anti-cancer agent currently in clinical trials. Furthermore, fenretinide is a known inducer of cell death in many types of cancer cells, but the mechanism of its action was not well understood (39). Previously published findings have attributed fenretinide's cytotoxicity to the activation of reactive oxygen species and/or increases in ceramide biosynthesis (15-17,40). However, the results presented here provide an additional and/or alternative mechanism to explain fenretinide's cytotoxic mode of action. Previously, tandem mass spectrometric analysis, as well as, *in vitro* assays revealed that treatment of MCF7 cells with fenretinide increased dihydroceramide species, through the inhibition of dihydroceramide desaturase (2), leading us to question dihydroceramide's published biological inactivity (18) and prompting us to explore cell regulation mechanisms in which dihydroceramide may participate. Biophysically speaking when compared to ceramides, dihydroceramides have a lower dipole potential which results in a decrease packing density within the membrane (41). Also, dihydroceramides have a lesser tendency to promote flip-flop or trans bilayer movement within the membrane (42), and dihydroceramides have been shown to be less likely to form and in some cases inhibitory to membrane channel formation when compared with ceramides (43). These differences may explain why dihydroceramide does not share many signaling pathways with ceramide, but in spite of these differences, this research has identified one cellular process that does allow for participation from both ceramide and dihydroceramide – the formation of cytoplasmic vacuoles known as autophagosomes as evidenced by GFP-LC3 confocal analysis (Figure 3).

In addition to identifying a previously uncharacterized signaling function for dihydroceramide, our study has provided the first evidence of a novel autophagic pathway induced by fenretinide, differing from previous studies which cited fenretinide's cell death mechanism via induction of apoptosis(15-17). The idea must not be excluded that fenretinide-induced autophagy may be occurring simultaneously with the apoptosis observed in other studies. However, further evidence is needed to confirm this idea, and we hypothesize that if both do occur as a result of fenretinide treatment that the time scale for each cell death event is different, with apoptosis occurring on a more rapid time scale to eliminate the most sensitive cell populations and autophagy occurring on a slightly longer time scale to eliminate more resistant cell populations (44). Nevertheless, our results clearly indicate that MCF7 cells undergo autophagy in response to fenretinide treatment (Figure 4).

When exploring the mechanism of fenretinide induced autophagy, we discovered that *de novo* sphingolipid biosynthesis, notably the production of dihydroceramide, is required for both the cytotoxic and autophagic actions of fenretinide, as indicated by cell viability and confocal image analysis of fenretinide treated cells following pre-treatment with ISP1 (Figure 5). Furthermore, these results suggest that there is a strong possibility that this 4HPR-induced autophagy could lead to 4HPR-induced cell death. However, our results do not provide ample causation between the independent events of sphingolipid dependent fenretinide-induced autophagy and sphingolipid dependent fenretinide cytotoxicity, but the results do show a correlation between the two events that should be explored further.

Further exploration of fenretinide's mechanism of action involving dihydroceramide accumulation, revealed that the turnover of dihydroceramide also plays a key role in the fate of cells treated with fenretinide. The results in Figure 6 revealed that when acid ceramidase expression and dihydroceramide turnover was suppressed, fenretininde cytotoxicity decreased, suggesting that acid ceramidase plays a pivotal role in fenretinide-induced cell death in MCF7 cells through the production of a cytotoxic sphingoid base, sphinganine. Furthermore, these results implicate sphinganine as a key component in fenretinide induced lethal autophagy, through its ability to behave as a lysosomotrophic agent (12), which induce cell death by permeablizing the lysosomal membrane. This permeablization leads to the release of cell death activators, such as cathepsins, into the cytosol. These released proteases may then serve as upstream activators of cell death pathways (12).

Additionally, some of the sphinganine produced from the hydrolysis of dihydroceramide will efflux from the autophagolysosome in a manner analogous to the lysosomal recycling mechanism of sphingolipids becoming phosphorylated by sphingosine kinase to yield sphinganine 1-phosphate (13). This sphinganine 1-phoshate is then thought to stimulate a protective autophagic mechanism within the cell, consistent with a previously published study (35). Our results in Figure 7 provide further support for this notion displaying that when sphingosine kinase is inhibited by DMS, cell viability decreases much more quickly and to a much larger extent. The results suggest that the prevention of sphinganine 1-phosphate formation blocks the protective autophagic mechanism and promotes the lethal autophagic mechanism by disturbing the delicate ratio of toxic sphinganine to protective sphinganine 1-phoshate. It is our hypothesis that

fenretinide's cytoxicity is not due to the inhibition of sphingosine kinase but rather to insufficient activity of sphingosine kinase to handle the large accumulation of sphinganine resulting from the turnvover of dihydroceramide within the autophagolysosome. Figure 8 provides a summation of the results of this study and our current working model of fenretinide's mechanism of action within the cell.

In conclusion, a myriad of recent hypotheses speculate and predict the relationship between autophagy and cell death (24,32). The term lethal autophagy used throughout this paper does not attempt to differentiate between apoptotic and autophagic cell death. In our studies, lethal autophagy simply implies that cells contain autophagosomes prior to undergoing cell death, and we are currently unsure if this cell death is caspase-dependent or caspase-independent. A study by Lamparska-Przybysz et al. suggests that the release of cathepsin B, a protease located in the autophagolysosome, serves as a molecular switch between autophagy and either apoptosis or lethal autophagy (44). The released cathepsin B cleaves BID and activates caspases, hallmarks of apoptosis (45). This idea of a molecular switch that regulates protective and lethal autophagy possibly provides an intriguing explanation for the ability of a cell to use autophagy as both a cell survival and cell suicide pathway. With respect to our study, monitoring cathepsin localization might provide insight into the ability of alterations in the ratio of sphinganine and sphinganine-1-phosphate to serve as a molecular switch regulating protective and lethal autophagy in 4HPR treated MCF7 cells. Our future goal is to further understand the complexities of the molecular basis of fenretinide's role in cell death via this novel autophagic pathway in MCF7 cells.

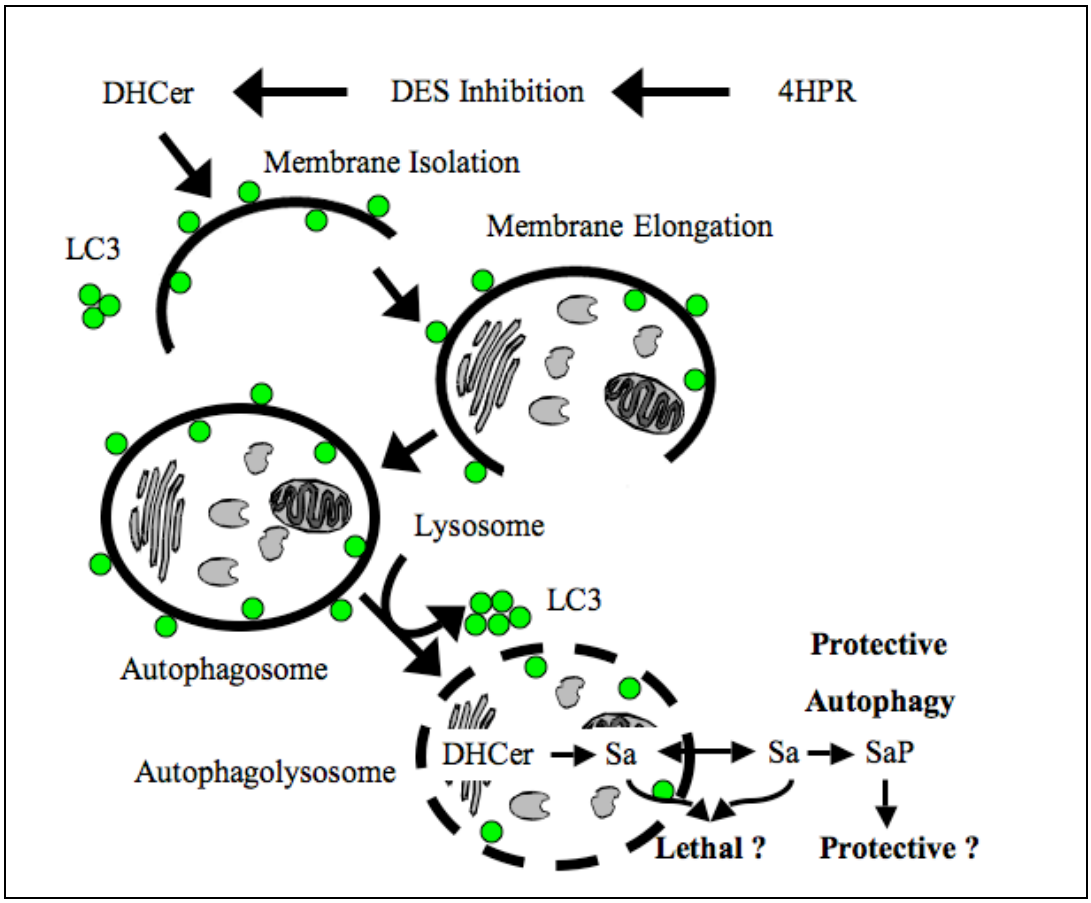


Figure 8. Proposed mechanism of fenretinide induced autophagy. It is our working hypothesis that fenretinide induced dihydroceramide accumulation results in the formation of autophagosomes. After formation, the autophagosome fuses with lysosomes where it acquires lysosomal sphingolipid hydrolyases, including acid ceramidase. Presumably, the acid ceramidase (AC) will hydrolyze some of the accumulated dihydroceramide to sphinganine. The sphinganine has two possible fates: 1) It can efflux from the autophagolysosome in a manner analogous to the process that occurs during lysosomal degradation of sphingolipids and become phosphorylated (which could result in protective autophagy through the formation of sphingosine 1-phosphate (35)), or 2) Sphinganine can accumulate in the autophagolysosome due to insufficient sphingosine kinase activity resulting in autophagolysosomal membrane destabilization because of its known ability to behave as a lysosomotrophic agent (12), which in turn results in the release of pro-death molecules activating cell death pathways.
Fenretinide (4HPR), Dihydroceramide (DHCer), Sphinganine (Sa), Sphinganine-1-Phosphate (SaP).

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